INVESTIGATION OF THE EFFECT OF QUERCETIN ON NEUROLOGICAL ALTERATIONS DURING TYPE 2 DIABETES MELLITUS IN SWISS ALBINO MICE

SYNOPSIS

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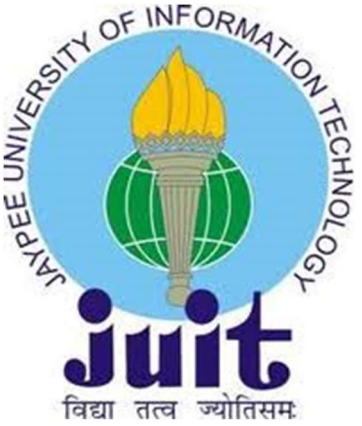

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Abstract

Diabetes mellitus (DM) is a complex progressive metabolic disorder arising from variety of pathogenic mechanisms, genetic or environmental, resulting in hyperglycemia. Hyperglycemia leads to both acute symptoms and chronic metabolic abnormalities, amongst which neuropathy, is one of the major causes of morbidity during DM. In the present study, we screened 267 small molecules of natural origin for their potential against diabetes and associated neurological complications through docking studies, followed by *in-vitro* assays. Our results demonstrated quercetin to be most efficient molecule and hence we further investigated its potential to modulate diabetes and neurological complications associated with long standing diabetes through *in-vivo* experimental models. To induce experimental type II diabetes mellitus (T2DM) we used multiple low dose streptozotocin (STZ) injection model in mice and investigated the effect of quercetin treatment on T2DM and associated neurological complications. Further, to get a better insight into the mechanism of quercetin action, we induced insulin resistant state in mice by subjecting them to 21 day chromic unpredicted stress (CUS) and investigated the neuromodulatory effect of quercetin treatment.

T2DM was induced in Swiss albino mice (20-25 g) by injecting 50 mg/kg STZ for 5 consecutive days. Diabetes was allowed to progress for 8 weeks during which animals were treated with 15 mg/kg quercetin and 5 mg/kg rosiglitazone in the treatment groups. STZ treated animals showed steady increase in the blood glucose levels, development of glucose intolerance and insulin resistance (IR). Diabetic animals were highly anxious, depressed and showed marked learning and memory dysfunction. Quercetin and rosiglitazone treatment improved hyperglycemia, glucose intolerance, attenuated IR and behavioral dysfunction. Behavioral deficits in diabetic animals were attributed to hyperglycemia mediated enhanced hippocampal oxidative stress, neurodegeneration and impaired neuronal insulin signaling. Quercetin and rosiglitazone treatment alleviated hippocampal oxidative stress, improved neuronal morphology, enhanced neurogenesis and upregulated insulin signaling pathway, especially in the CA3 region of the hippocampus.

Further, we induced IR in Swiss albino mice by subjecting them to 21 day CUS paradigm and investigated the neuroprotective potential of quercetin in it. CUS induced pre-diabetic state, IR and behavioral dysfunctions, which were associated with enhanced hippocampal oxidative stress, neurodegeneration and impaired hippocampal insulin signaling. 21 day quercetin treatment (30

mg/kg; *po*; *od*) during CUS improved glucose homeostasis, alleviated IR and behavioral dysfunctions by attenuating hippocampal oxidative stress, enhancing neurogenesis and by improving insulin signaling pathway.

Further, we confirmed the mechanism of quercetin action through cell line based studies. We cultured SH-SY5Y neuroblastoma cells with quercetin and insulin in the presence and absence of insulin signaling inhibitor, LY294002, and determined the glucose uptake through fluorescent glucose (2NBDG) uptake assay. Our results demonstrated that in the absence of LY294002, both quercetin and insulin markedly enhanced glucose uptake. Glucose uptake was significantly reduced after treating cells with LY294002. These results suggests that quercetin exploits insulin signaling pathway to exert its pharmacological effects. To determine whether quercetin binds to InR or not, we used *in-silico* docking studies. Results suggest that quercetin is having a strong interaction with the InR, however, binding site is different from insulin binding site.

Mechanistically, quercetin upregulated the neuronal insulin signaling pathway and enhanced GLUT4 expression, independent of insulin and InR. In conclusion, quercetin may find a clinical application in managing neurological complications associated with T2DM and IR by activating neuronal insulin signaling pathway, irrespective of insulin or InR expression.

Introduction

Diabetes mellitus (DM) is a complex metabolic disorder arising from variety of pathogenic mechanism, genetic or environmental, resulting in hyperglycemia. Hyperglycemia leads to both acute symptoms and chronic metabolic abnormalities, amongst which neuropathy, nephropathy, retinopathy and cardiovascular diseases are the major causes of morbidity during DM (1). Hyperglycemia may be a result of pancreatic β-cells destruction thereby decreasing the level of insulin secretion (Type-1 Diabetes Mellitus; T1DM) or due to reduced responsiveness of target cells towards insulin, with or without insulin secretory defects (Type-2 Diabetes Mellitus; T2DM) (2). Clinically, polydipsia, polyuria, polyphagia, weight loss, fatigue and numbness in the extremities are the major symptoms of DM (2, 3). Globally, 422 million adults were diagnosed positive for diabetes in the year 2014 accounting for a prevalence of 8.5% among the adult population. It is estimated that about 46% cases of T2DM remain clinically undiagnosed. Prevalence of DM has quadrupled since 1980 and is expected to increase up to 642 million by the year 2040. Diabetes is the 6^{th} leading cause of death globally accounting for 1.5 million deaths directly, besides, higher-than-optimal blood glucose was associated to additional 2.2 million deaths as a result of its secondary complications of DM, for a total of 3.7 million deaths related to blood glucose levels. India shares second maximum load of DM in the world, accounting for 69.2 million people (8.7%) and remains undiagnosed in more than 36 million people (4). The prevalence of diabetes and its risk factors are increasing at an alarming rate, in both, developed and developing nations. Obesity, overweight and lack of physical activity are the major risk factors for the development of hyperglycemia. Globally, more than 1 in 3 adults were observed to be overweight, more than 1 in 10 were categorized as obese and 84% of girls and 78% of boys were short of minimum requirements for physical activity for their age (4). These disturbing figures clearly indicate the need for looking for the counter measures against DM and its associated secondary complications.

DM is associated with several secondary complications, such as neuropathy, nephropathy, retinopathy, cardiovascular complications etc., which are primarily responsible for the mortality and morbidity associated with DM, besides, significantly deteriorating the quality of life. Though many medications are available today for the management of diabetes, they only provide symptomatic relief by reducing blood glucose levels, without modulating the causative factors of DM or its complications. Neurological complications of diabetes are highly devastating and are

least understood. Despite of enormous healthcare advancement and regular antidiabetic therapy, these neurological complications keep on progressing day by day, that extremely impair the quality of life. Thus, demand of the present day is to search for some suitable alternatives which could not just halt the progression of diabetic but also attenuate the central nervous system (CNS) abnormalities that have already been set in.

Diabetes is known to cause many structural, chemical and functional abnormalities in the CNS. Neurodegeneration, memory impairment, neurochemical changes, structural changes in blood brain barrier (BBB), hippocampus and cortex and ultrastructure abnormalities in mitochondria, endoplasmic reticulum, microtubule and chromatin mainly contributes to central diabetic (5). Mechanisms leading to diabetic neuropathy are linked to reactive oxygen/nitrogen species (ROS/RNS) generation, either directly or indirectly, followed by increased oxidative stress, mitochondrial dysfunction and inflammation. Hyperglycemia increases the load of ROS/RNS through advanced glycation end products (AGE), polyol, hexosamine, PkC, PPAR and oxidative stress pathways leading to saturation of antioxidant defense of our body, which are responsible for neutralization of these free radicles. ROS/RNS in turn leads to the destruction of neurons, especially axon where blood supply and mitochondria are present in abundant amount (6-8). Hippocampus region of the brain is known to be involved in learning and memory functions. Further, regulation of neurogenesis interferes with the learning and memory function of the individual (9). Recently reported studies associates both, T1DM and T2DM with reduced hippocampal neurogenesis (10-13). Role of peripheral insulin in brain is controversial, some studies conclude that peripheral insulin easily crosses BBB and other states that insulin is indigenously synthesized in the brain (14-17). Brain consist of 2% of the total body weight and consumes upto 70% of total glucose utilized in body. Hippocampus and cortex are rich in insulin receptors (InR), which on activation by insulin enhance expression of insulin responsive glucose transporter (GLUT4) necessary for maintaining energy homeostasis of the neuron. In side CNS, insulin is degraded by insulin degrading enzyme (IDE), which is also known to clear amyloid-β (Aβ) from the brain. During T2DM, high blood glucose level induces hyperinsulinemia which downregulates insulin uptake by BBB thereby reducing its concentration in the brain. Lack of insulin downregulates IDE and thus Aβ is gets accumulated in the brain. Aβ deposits in hippocampus forming amyloid plaques leading to neurodegeneration, decline in cognitive functions, dementia and late onset Alzheimer's disease (LOAD). Further, insulin resistance (IR)

developed during T2DM lowers glucose uptake into the neurons, leading to neuronal starvation, degeneration and alterations in behavioral and CNS functions. In T2DM all these factors combine together and contribute towards the decline in cognitive functions (18, 19).

Stress is an essential defense mechanism which physically and mentally prepares an individual to cope up with the stressful situations in the daily life. However, chronic and prolonged stress has deleterious effect on the body, especially in the CNS. Chronic stress results in prolonged activation of hypothalamic-pituitary-adrenal (HPA) axis, causing adrenal hypertrophy and excessive production of glucocorticoid. Glucocorticoid freely crosses BBB and promotes hippocampal atrophy and functional decline by lowering glucose uptake and ATP generation, elevating oxidative stress and by impairing hippocampal glutamate reuptake from synapse resulting in prolonged excitotoxicity (20). More importantly, hippocampus has high density of corticosterone receptors and therefore, hippocampal neurons are highly vulnerable to glucocorticoid toxicity. Chronic stress impairs hippocampal neuronal plasticity and induces hypercorticosteromia, neurodegeneration and behavioral dysfunction such as depression, and cognitive dysfunction (21-25). Other than these central complications, hypercorticosteromia impairs glucose homeostasis and induces type-2-diabetes-like state by lowering insulin secretion from pancreatic β-cells, reducing glucose uptake/utilization, and inflicting IR (26-30). It is now evident from the recent findings that, both chronic stress and T2DM are actively associated with the development of memory impairment, cognitive dysfunction, and Alzheimer's disease (29-33). In a separate study, it was reported that impaired cognitive function, characterized by low episodic memory performance, precedes stress and depression and might be considered as a premorbid marker of depression (34). Glucocorticoid further impairs cognitive function by elevating the Aβ protein level in hippocampus, reducing size of hippocampus, prefrontal cortex and other areas responsible for mood and cognition (35-37). Further, hypercorticosteromia has been reported to promote fat deposits viscerally which in turn cause inhibition of InR and affects insulin sensitivity levels in such patients (38) causing IR, and thus T2DM by antagonizing the hypoglycemic effect of insulin (39).

Brain needs continuous and uninterrupted glucose supply to maintain its function and to maintain glucose homeostasis throughout the body. Insulin is a peptide hormone which binds to InR and is known to play a vital role in key biological processes that are necessary for normal growth,

development and functioning of the body. In the past few decades extensive research on insulin reports its presence in CNS. InR are expressed in large number in neurons, glial cells, cerebral cortex, hippocampus, hypothalamus, and olfactory bulb i.e. areas that modulate behavior, reproduction, learning, memory and cognition (40). However, the exact role and mechanism by which it operates in CNS still remains unclear to large extent. Also, locally synthesized insulin in brain has been reported (41, 42). Insulin/insulin signaling has neuromodulatory and neurotropic effect on the brain and is necessary for maintaining normal cognitive functioning, learning & memory and synaptic plasticity (43-45). Abnormal insulin/ InR activities are associated with cognitive dysfunction (46) and chronic stress (38). Further, central IR impairs neuronal plasticity and intensifies neuronal oxidative stress (47), cognitive dysfunction (48) and enhance vulnerability for neurodegenerative disorders such as Alzheimer's disease (49). Furthermore, hippocampal expression of insulin dependent GLUT4 on neurons is necessary for learning and memory acquisition (50).

Keeping in mind the side effects and non-availability of clinical anti-diabetic therapeutic to prevent or reverse the progression of neurological complications associated with diabetes, research work in the recent years has been focused on finding an alternative and safe therapeutic strategies, which not only prevent the progression of T2DM, but also alleviate the complications that had already set in. Recently, WHO reported that 90% cases of T2DM can be prevented by making necessary lifestyle and dietary changes (51), indicating the impact of herbal therapeutic and dietary management of DM. Flavonoids are regularly consumed in diet in the form of fruits, vegetables, beverages etc., and are well known antioxidants. Clinically, enhanced dietary intake of flavonoids is known to lower the risk of T2DM development and inhibit oxidative DNA damage in the diabetic patients (52, 53). Plants and plant preparation such as *Gingo biloba* (54), sweetened dried and raw cranberries (55), green tea, grapes (56, 57), *Dodonaea viscosa* (58), *Cecropia pachystachya* (59), *Vaccinium angustifolium* (60), *Helichrysum plicatum* (61), *Lamiaceae sp.* (62), *Urtica dioica* (30) and *Psidium guajava* (63), which are rich source of flavonoids, are reported to possess antidiabetic potential in various animal models.

Objectives of the study

- To screen small molecules of natural origin through *in-silico* docking studies and *in-vitro* assays for the management of diabetes and associated neurological complications.
- To evaluate the effect of quercetin on diabetes and associated neurological complications during STZ mediated diabetes in Swiss albino mice.
- To evaluate the effect of quercetin on insulin resistance in an experimental model of chronic unpredicted stress.
- To elucidate the mechanism through which quercetin modulates glucose homeostasis

Objective 1

To screen small molecules of natural origin through *in-silico* docking studies and *in-vitro* assays for the management of diabetes and associated neurological complications

In-silico docking studies

Docking studies were used to predict molecules with potential to be beneficial in diabetes and associated neurological complications. Based on extensive literature survey, 267 small natural molecules were identified which were reported to be potentially bioactive during diabetic state. These molecules were subjected to docking simulation studies against InR (PDB id: 1IR3), peroxisome proliferator-activated receptor-γ (PPAR-γ) (PDB id: 2PRG) and dipeptidyl peptidase-4 (DPP4) (PDB id: 4PNZ) using AutoDock (4.2.6) software. Results were expressed in terms of free energy (kcal/mol; docking score) of ligand-protein interaction. We observed minimum ligand-protein interaction energy for ascorbic acid, gallic acid, quercetin, caffeine, ellagic acid, piperine and cinnamic acid on all three docked proteins. Based on these findings, these molecules were predicted to have most efficient interaction with InR, PPAR-γ and DPP-4, and were subjected to narrowing down screening process through *in-vitro* screening.

In-vitro screening studies

Screening process was further narrowed down by subjecting ascorbic acid, gallic acid, quercetin, caffeine, ellagic acid, piperine and cinnamic acid through *in-vitro* assays against diabetes and various pathways which leads to the development of diabetic complications and the results were compared with marketed anti-diabetic drugs, rosiglitazone (ROSI), metformin and glimepiride. Comparative *in-vitro* antioxidant activity of the selected natural small molecules and marketed anti-diabetic drugs were evaluated through 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay (64) and inhibition of lipid peroxidation assay (65) and genotoxicity was evaluated through comet assay (66). Further, we evaluated comparative anti-diabetic potential through inhibition of α-amylase activity and evaluated the effect of these molecules on neuronal toxicity, neuronal glucose uptake and GLUT4 expression in primary hippocampal culture prepared from E18 rat embryo. Our results demonstrated ascorbic acid, gallic acid, quercetin and ellagic acid to be potent antioxidants in dose dependent manner. IC_{50} values were observed to be in the range of 0.006 - 0.03 μM for the DPPH radical scavenging and 0.92 - 1.07 μM for the inhibition of lipid peroxidation. Caffeine, piperine, cinnamic acid, rosiglitazone, metformin and glimepiride

did not show any promising antioxidant potential and their activity was found to be significantly lower than ascorbic acid, gallic acid, quercetin and ellagic acid at the tested concentrations. Oxidative stress plays a critical role in the development of diabetes associated neurological complications (7). It is now evident that reduction in oxidative stress is associated with improving neurological functions and benefits cognitive impairments, depression, anxiety, Alzheimer's disease etc. (31, 67-69). Herein, we demonstrated that ascorbic acid, gallic acid, quercetin and ellagic acid efficiently scavenged free radical and prevented lipid peroxidation. Thereby, these molecules may be beneficial during oxidative stress mediated neurological alterations. Further, ROSI, metformin and glimepiride were ineffective against oxidative stress, which possibly is the reason for the uncontrolled progression of diabetes mediated neurological complications, despite of regular therapeutics. Supplementing diabetic therapeutics with natural antioxidants may aid in coping up with diabetes associated neuropathy.

Genotoxicity is a morbid condition, which is associated with diabetes and is known to exaggerate diabetes mediated neurological complications (70, 71), and therefore interventions which are capable of managing genotoxicity may also benefit neurological complications. In our study, we observed that quercetin, gallic acid, piperine and caffeine efficiently alleviated hydrogen peroxide (H_2O_2) induced DNA fragmentation as olive tail movement was significantly reduced after treating cells with these molecules. Tested marketed drugs did not provide any protection against genotoxicity and significantly higher tail length in comet was observed, when compared to control. These results suggest that these drugs cannot provide any protection against genotoxicity and therefore neuronal dysfunction. Quercetin, gallic acid, piperine and caffeine possesses high potential to alleviate genotoxicity and thereby may be capable of rescuing neurons form genotoxicity mediated damage observed during long standing diabetes.

α-amylase is a key enzyme involved in the carbohydrate metabolism and is known to enhance dietary glucose uptake, and thereby elevates blood glucose level. Inhibition of this enzyme alleviates hyperglycemia by reducing dietary glucose influx (64, 72). Results of a comparative study for natural molecules against α-amylase activity revealed that quercetin (IC_{50} = 18.31 μM) and ellagic acid (IC_{50} = 12.49 μM) efficiently inhibited α-amylase activity and a percent inhibition of 85.83 ± 0.83% and 81.11 ± 1.27% respectively was observed at 500 μM concentration. These

results indicates that dietary supplementation of quercetin and ellagic acid can provide additional advantage in managing hyperglycemia by lowering dietary glucose input.

Further, we evaluated the neurotoxic effect of test molecules on primary cultures derived from E18 rat embryo using MTT assay. None of the compounds tested showed any toxicity towards neurons at 10 μM concentration and therefore further experimentation was carried out at this concentration. Our results suggested that natural molecules, especially quercetin and caffeine, significantly enhanced glucose uptake into neuronal cells, which was comparable to that observed for ROSI and metformin. Further, quercetin and caffeine resulted in enhanced GLUT4 expression on the neuronal membrane, which might be responsible for the enhanced glucose uptake demonstrated by these compounds. Results of the GLUT4 expression for quercetin and caffeine did not vary significantly from ROSI. Diabetes is characterized by persistent hyperglycemia, and thus any drug which is capable of disposing glucose from the circulation can be considered as an efficient antidiabetic moiety (72, 73). Our results clearly demonstrate that quercetin and caffeine can modulate neuronal glucose uptake by enhancing GLUT4 translocation. Enhanced expression of GLUT4 in the neurons is known to be associated with improved neuronal plasticity, memory performance and other neurological functioning (30, 31).

Conclusion

It is evident from the results obtained from *in-silico* and *in-vitro* screening that natural molecules, especially quercetin, might be beneficial in the management of neurological complications during diabetes by alleviating oxidative stress, genotoxicity, inhibiting α-amylase activity and more importantly by enhancing GLUT4 mediated glucose uptake into neuronal cells.

Objective 2

To evaluate the effect of quercetin on diabetes and associated neurological complications during STZ mediated diabetes in Swiss albino mice

Experimental design

Swiss albino mice (20-25 g; 18-20 week old) were used in the entire study. All the experiments were performed with prior approval from Institute Animal Ethics Committee (IAEC) as per guidelines of CPCSEA, India. All the necessary efforts were made to minimize suffering to the animals. Animals were divided into two groups, Group I: Control (n = 30) and Group II: Diabetic (n = 39). Animals in the group II were given 50 mg/kg intraperitoneal Streptozotocin (STZ) injection for 5 consecutive days (in ice cold citrate buffer, pH 4.5) between 7 and 8 am daily. Development of diabetes in these animals was confirmed by recording 12 h fasting glucose levels. Blood was taken from tail vein and animals having fasting blood glucose < 150 mg/dl were excluded from the study and animals with fasting blood glucose ≥ 150 mg/dl were considered as diabetic and were divided into three sub groups (n = 8-10), Group II (a): STZ + vehicle (0.3% carboxy methyl cellulose) (STZ), Group II (b): STZ + Quercetin (STZ + Q) and Group II (c): STZ + Rosiglitazone (STZ + ROSI). Animals in the group I were given weight equivalent ice cold citrate buffer through intraperitoneal injection for 5 consecutive days and were divided into three subgroups (n = 8), Group I (a): Control + vehicle (CTRL), Group I (b): Control + Quercetin (CTRL + Q) and Group I (c): Control + Rosiglitazone (CTRL + ROSI). Quercetin was administered at the dose of 15 mg/kg and ROSI at 5 mg/kg. All the treatments were given orally, once daily between 5-6 pm, for 8 weeks.

Development and progression of diabetes

Development and progression of diabetes was monitored symptomatically by evaluating body weight, food and water intake, fasting blood glucose and oral glucose tolerance test (OGTT). STZ induced stable diabetes in animals, which progressed steadily throughout the study. Symptomatically, feed intake and water intake was significantly ($p < 0.001$) elevated and body weight was significantly ($P < 0.01$) reduced in diabetic animals. Further, fasting blood glucose was significantly higher ($p < 0.001$) in diabetic animals, when compared to control group. Results of the OGTT confirmed the development of glucose intolerance in diabetic animals as the blood glucose at each time point remained significantly higher ($p < 0.001$) than control animals.

Polyphagia, polydipsia hyperglycemia and elevated OGGT are the characteristic symptom of T2DM, and development of these symptoms in diabetic animals confirmed the successful induction of diabetes, which resembled clinical T2DM symptomatically. Treating diabetic animals with quercetin attenuated the symptoms of diabetes. Water intake and fasting blood glucose levels were significantly ($p < 0.05$) reduced after 8 week of quercetin treatment. Body weight was normalized, however, in the first two weeks of the study animals treated with quercetin showed reduction in body weight, which was normalized by 5^{th} week of the study. Further, results of OGGT demonstrated that quercetin effectively alleviated glucose intolerance as the blood glucose was significantly ($p < 0.001$) lower than STZ. Interestingly, control animals treated with quercetin showed lower glucose levels than CTRL animals, indicating that quercetin treatment in normal animals did not allowed blood glucose to rise. These results suggest that quercetin is having potential to dispose blood glucose in normal as well as in diabetic animals. Further, treating diabetic animals with ROSI resulted in an insignificant improvement in feed intake and body weight, however it significantly ($P < 0.05$) alleviated polydipsia in diabetic animals. Although, in OGTT test STZ + ROSI showed significantly lower blood glucose levels, quercetin treatment was observed to be more efficient in managing glucose intolerance.

Behavioral studies

Effect of quercetin treatment on diabetes induced behavioral dysfunction was evaluated after 8 weeks of the treatment. Locomotor activity was evaluated by using Actophotometer and open field test (OFT) (74). Muscle coordination was evaluated by using Rota-rod apparatus and beam walk test (74). Anxiety level in experimental animals was evaluated through elevated plus maze (EPM) and OFT (74). Depressive behavior was evaluated through sucrose preference test (SPT), tail suspension test (TST) and forced swim test (FST) (74). Memory functions were evaluated through passive avoidance step down and step through (PA-SD; PA-ST) task, novel object recognition (NOR) test and Morris Water Maze (MWM) test (74).

Long-standing diabetes resulted in reduced locomotor activity, as indicated by lower number of line crossings in Actophotometer and OFT ($P < 0.001$). Treating animals with quercetin and ROSI improved locomotion and animals were observed to freely explore the test field. Likewise, the results of the Rota-rod test and beam walk demonstrated nonsignificant difference amongst different groups, however time taken to fall from rotating arm of Rota-rod was lower in STZ and

diabetic animals took comparatively greater time to cross 1 meter long beam. These results suggest that diabetes induced locomotor dysfunction, which may be attributed to diabetes mediated impairment in CNS functions, since muscle coordination and muscle strength were observed to be normal in the diabetic group.

STZ treatment induced marked anxiety in the animals as indicated by the significantly ($P < 0.001$) lesser time spent in the open arm of the EPM. Our results demonstrated that diabetes significantly ($P < 0.001$) reduced time spend in the open arm of the EPM. Treating diabetic animals with quercetin improved exploration behavior and these animals spent significantly ($P < 0.001$) greater time exploring the open arm of EPM, when compared to STZ. Treating diabetic animals with ROSI (STZ + ROSI) significantly alleviated anxiety ($p < 0.001$), however, effect of quercetin treatment was observed to be more pronounced than ROSI treatment. We further evaluated anxiety behavior of animals inside OFT, in terms of number of center entries and time spent in the central region of OF. STZ treatment significantly ($p < 0.001$) elevated anxiety levels in the animals, diabetic animals rarely entered the central region and spent lesser duration of time in the central region of the OFT. Anxiolytic effect of quercetin treatment was confirmed through OFT and STZ + Q animals showed significantly ($P < 0.001$) higher number of entries to the central region and spent significantly ($p < 0.001$) greater duration of time there, when compared to STZ. Treating diabetic animals with ROSI did not produced any significant effect on the time spent in the central region of the OF, although, a significantly ($P < 0.05$) higher entries to the central region was observed, when compared to STZ. These results suggests that quercetin is a potent anxiolytic moiety and it efficiently alleviated diabetes mediated anxiety disorder in mice.

We further evaluated the effect of quercetin on diabetes mediated depressive like behavior. We observed that diabetes resulted in significant ($p < 0.001$) anhedonia, and animals showed lack of interest towards rewarding stimuli. Depressive like behavior in diabetic animals was further confirmed by TST and FST, where animals showed significantly ($p < 0.001$) higher duration of immobility. Treating animals with quercetin and ROSI alleviated STZ mediated anhedonia and significantly ($p < 0.05$) reduced immobility duration during TST and FST. Moreover, the effect of quercetin treatment was observed to be more pronounced than ROSI. These results suggest that quercetin could efficiently alleviate diabetes mediated depression.

We further investigated the effect of quercetin treatment on short term and long term memory retention during long standing diabetes through PA-ST and PA-SD task. Diabetic animals showed significantly impaired memory performance in PA-SD and PA-ST task. Memory acquisition training was given on day 1 and memory retention was evaluated on day 2 (short term memory) and day 5 (long term memory). Inflexion ratio in PA-SD and PA-ST was observed to be significantly ($p < 0.01$) lower on day 2 and day 5 in diabetic animals, suggesting that diabetes adversely affect memory retention in mice. Further, quercetin treatment alleviated memory dysfunction in diabetic animals as evident from the significantly ($p < 0.001$) higher inflexion ratio of day 2 and day 5, when compared to STZ group. Results of STZ + ROSI revealed a significant ($p < 0.05$) improvement in the short term and long-term memory function in PA-SD and PA-ST task, as evident from the significantly higher inflexion ratio, when compared to STZ. Quercetin treated diabetic animals demonstrated higher inflexion ration than ROSI treated animals, suggesting that quercetin is more efficient than ROSI in alleviating memory dysfunction. We further evaluated the effect of quercetin treatment on alterations in recognition memory using NOR test. Diabetic animals spent almost equal time exploring both, novel and familiar objects, indicating that they were not able to recognize the familiar object. Discrimination index was significantly ($P < 0.001$) reduced in diabetic animals, and quercetin and ROSI treatment efficiently ($p < 0.001$) improved memory performance, as animals were observed to spend longer duration exploring the novel object. We further evaluated the effect of quercetin treatment on diabetes mediated learning and working memory dysfunction using MWM test. On day one of training, all the animals struggled to find the hidden platform and transfer latency did not differed significantly in all the treatment groups. Effect of diabetes on learning abilities of animals became evident from second day of training where diabetic animals showed significantly ($P < 0.001$) higher transfer latency when compared to CTRL. On day 3 and day 4, transfer latency in STZ group were observed to be significantly ($P < 0.001$) higher than CTRL group. Quercetin and ROSI treated animals showed good learning across the test and transfer latency was observed to be significantly ($P < 0.05$) lower than STZ and were comparable to CTRL. We now evaluated the memory index in term of total time spent in the platform quadrant and number of platform crossings (probe trial). Results revealed the STZ animals spent significantly ($P < 0.001$) lower time in platform quadrant and showed significantly ($P < 0.05$) lower number of platform crossings. Quercetin and ROSI treated animals showed good memory index and animals spent significantly ($P < 0.001$) higher

time in platform quadrant and crossed platform position frequently ($P < 0.05$) in search of escape platform. These results suggests that quercetin can efficiently rescue animals from diabetes mediated memory dysfunction.

Animal sacrifice and sample collection

Samples for the biochemical evaluations were collected 24 h after the last behavioral test. Animals were sacrificed under anesthesia (90 mg/kg ketamine and 5 mg/kg xylazine) and entire brain was either fixed in 2% paraformaldehyde and 2% glutaraldehyde solution (for histopathological and immunofluorescence studies) or used as such for Golgi-cox staining. For biochemical and proteomics studies, animals were sacrificed by cervical dislocation, hippocampus was dissected over ice, homogenized in RIPA buffer and stored at -80ºC.

Biochemical evaluation

Blood glucose levels were measured by tail vein puncture by using Accu-check blood glucose monitoring system, serum insulin levels were determined by using commercial assay kit (AccuLite CLIA Microwells, Monobind Inc., USA), homeostasis model assessment-estimated insulin resistance index (HOMA-IR) was determined using fasting blood glucose (mmol/L) and insulin levels (mU/L). Serum corticosterone levels were quantified by using HPLC-UV system (30). Effect of quercetin treatment on hippocampal oxidative stress markers was evaluated in the hippocampal homogenate in terms of total thiol levels (TTH) (412 nm), thiobarbeturic acid-reactive substances (TBARS) (540 nm), nitric oxide (NO) (540 nm) and catalase levels (240 nm) as per our previously described method (75).

STZ treatment resulted in significant ($P < 0.001$) hyperglycemia, which steadily progressed throughout the study, thereby indicating successful induction of long standing diabetes in experimental animals. Quercetin and ROSI treatment efficiently controlled hyperglycemia and significantly ($P < 0.001$) lowered blood glucose levels in diabetic animals. Further, diabetic animals showed significantly ($P < 0.05$) lower serum insulin levels, when compared to CTRL. Insulin levels in the diabetic animals treated with quercetin and ROSI were also observed to be lower than CTRL and did not vary significantly from STZ. Lower insulin levels in the diabetic animals may be attributed to the damaging effect of STZ on insulin producing pancreatic islet cells. Interestingly, normal animals treated with quercetin showed nonsignificantly lower serum insulin levels, when compared to CTRL, suggesting that quercetin might have played some direct

role in lowering blood glucose levels. Further, we successfully demonstrated the development of T2DM in experimental animals through HOMA-IR index, which is the marker of IR. Diabetic animals showed significantly ($P < 0.001$) higher HOMA-IR index and quercetin treatment efficiently ($P < 0.05$) lowered it, suggesting that quercetin might be beneficial in managing IR and thus T2DM. ROSI treatment also resulted in a significant ($P < 0.01$) reduction in HOMA-IR index, which may be attributed to its potential to alleviate IR and increase insulin sensitization (7, 72).

We further demonstrated that STZ-induced diabetes inflicts severe oxidative stress, as indicated by the significantly ($P < 0.001$) elevated TBARS and NO levels and significantly ($P < 0.001$) reduced TTH and catalase levels in the hippocampus of diabetic animals. Treating animals with quercetin alleviated oxidative stress, as evident from the significant ($P < 0.01$) reduction in oxidative stress markers (TBARS and NO) along with significantly ($P < 0.001$) elevated antioxidant defense (TTH and catalase) markers. ROSI treatment lowered hippocampal oxidative stress but the observed effects were less prominent than quercetin. Quercetin is a potent natural antioxidant, which might have played a direct role in rescuing animals from diabetes mediated oxidative stress. These results confirms our *in-vitro* results (objective 1), where quercetin was observed to be a potent antioxidant.

Neurodegeneration

Effect of quercetin treatment on diabetes induced hippocampal neurodegeneration was evaluated through Golgi-cox staining (76, 77). Our results demonstrated that diabetes inflicts severe damage in the hippocampus region of the brain, especially in the CA3 region. Quercetin and ROSI treatment rescued neurons from diabetes mediated damage and neurons resembled normal morphology. Moreover, Golgi-cox staining revealed that diabetes inflicted severe neurodegeneration, neurons were short, showed less network, appeared severely damaged and had significantly lower spike density. Quercetin treated neurons were healthy with numerous networking, good arborization and significantly ($p < 0.01$) higher spike density, when compared to STZ. Animals treated with ROSI were also observed to be healthy with significant ($P < 0.01$) arborization and significantly ($P < 0.01$) higher spike density.

Protein expression studies

In order to get a better insight into the effect of diabetes and quercetin treatment on hippocampal insulin signaling, protein expression in the hippocampus was determined through western blot

analysis (31). Diabetic animals showed significantly ($P < 0.001$) lower expression of insulin, InR and GLUT4 in the hippocampus, when evaluated through western blot analysis. Quercetin treatment significantly upregulated hippocampal expression of insulin ($P < 0.05$), InR ($P < 0.05$) and GLUT4 ($P < 0.001$). Moreover, ROSI treated animals also exhibited significantly higher expression levels of insulin ($P < 0.01$), InR ($P < 0.05$) and GLUT4 ($P < 0.01$). Interestingly, control animals treated with quercetin (CTRL + Q) demonstrated nonsignificant downregulation of hippocampal expression of insulin, significantly lower ($P < 0.01$) InR expression and significantly ($P < 0.01$) higher GLUT4 expression, when compared to CTRL. These results suggest that quercetin is capable of upregulating neuronal GLUT4 expression in normal as well as diabetic animals, despite of lower InR and insulin levels in the hippocampus. Moreover, diabetes significantly ($P < 0.001$) reduced hippocampal neurogenesis, which was significantly upregulated by quercetin ($P < 0.001$) and ROSI ($P < 0.05$) treatment. Interestingly, quercetin treatment significantly ($P < 0.01$) enhanced hippocampal neurogenesis and ROSI treatment significantly ($P < 0.05$) reduced hippocampal neurogenesis. These results suggest that quercetin can upregulate hippocampal neurogenesis in normal as well as diabetic condition. Further, in order to get a better idea of the mechanism of action of quercetin, we performed immunofluorescence studies. Expression of InR was significantly ($P < 0.001$) elevated and GLUT4 expression was significantly ($P < 0.001$) reduced in the CA3 region of the hippocampus during diabetes. Treating diabetic animals with quercetin significantly ($P < 0.01$) reduced InR expression and significantly ($P < 0.001$) enhanced GLUT4 expression. Further, we observed a slight reduction in the InR expression and significant ($P < 0.01$) increase in GLUT4 expression in the CA3 region of the hippocampus in CTRL + Q animals, when compared to CTRL. These results suggest that quercetin is having potential to upregulate neuronal insulin signaling and GLUT4 expression despite of lower InR levels. ROSI treated animals showed significant improvement in InR ($P < 0.001$) and GLUT4 ($P < 0.05$) expression. Since GLUT4 is the end product of InR activation, we calculated the GLUT4:InR ratio to get a better insight into the effect of quercetin on neuronal insulin signaling pathway. Interestingly, we observed a significant ($P < 0.001$) reduction of GLUT4:InR ratio in diabetic animals, which was demonstrated to be significantly higher in CTRL + Q ($P < 0.001$), STZ + Q ($P < 0.01$) and STZ + ROSI ($P < 0.05$) animals, when compared to CTRL and STZ. These results clearly demonstrates the potential of quercetin to enhance GLUT4 expression in the hippocampus, independent of the InR expression levels.

Conclusion

Long standing diabetes induces behavioral dysfunction which could be attributed to enhanced oxidative stress, neurodegeneration, reduced synaptic plasticity and development of insulin resistant state in the hippocampus, especially in CA3 region. Treating animals with quercetin alleviated hyperglycemia and IR. Quercetin treatment improved behavioral functioning which can be attributed to its potential to lower oxidative stress, improve synaptic plasticity and neuronal survival. Quercetin seems to be acting through insulin signaling pathway but independent of both insulin and InR. Quercetin can be efficiently used to manage IR and T2DM, as well as its associated neuronal complications.

Objective 3

To evaluate the effect of quercetin on insulin resistance in an experimental model of chronic unpredicted stress

Experimental design

Swiss albino mice (20-25 g; 18-20 week old) were used in the entire study. All the experiments were performed with prior approval from Institute Animal Ethics Committee (IAEC) as per guidelines of CPCSEA, India. All the necessary efforts were made to minimize suffering to animals. Animals were divided into four groups (n = 10-12), Group I: Control (CTRL), Group II: CTRL + Quercetin (CTRL + Q), Group III: Chronic Unpredicted Stress (CUS) and Group IV: CUS + quercetin (CUS + Q). Animals in Group III and IV were subject to an array of randomly selected unpredicted stress for 21 days, during which 30 mg/kg quercetin treatment was given orally between 7 and 8 am. CTRL and CUS received equal volume of vehicle orally for 21 days. Biochemical, behavioral and molecular studies were performed afterwards.

Biochemical evaluation and development of insulin resistance

Development of diabetes like state and IR was determined by evaluating body weight, food and water intake, blood glucose, OGTT, serum insulin level and HOMA-IR index. Besides, serum corticosterone level was determined to evaluate the effect of quercetin on chronic stress. Our results demonstrated that subjecting animals to 21 days CUS induced pre-diabetic state in animals. Symptomatically, CUS resulted in a significant ($P < 0.001$) reduction in body weight, which may be associated with a significant reduction ($P < 0.01$) in feed intake observed in stressed animals, besides, water intake did not showed any significant difference when compared to CTRL. In our study, CUS resulted in a significant ($P < 0.001$) increase in serum corticosterone levels, which was significantly reduced by quercetin treatment ($P < 0.01$), indicating the beneficial effect of quercetin during stressful conditions. Further, 21 day CUS significantly ($P < 0.001$) elevated fasting blood glucose and serum insulin levels, however, hyperglycemia was observed in the pre-diabetic range. HOMA-IR index confirmed the development of marked IR in stressed animals, which may be attributed to the development of pre-diabetic state in these animals. Further, to get a decisive conclusion on IR, OGTT was performed and the results confirmed the development of insulin resistant like state in stressed animals as blood glucose at each time point and area under OGTT curve in CUS remained significantly higher ($P < 0.001$), compared to CTRL. Treating stressed

animals with quercetin significantly improved body weight ($P < 0.001$), feed intake ($P < 0.05$), fasting blood glucose levels ($P < 0.001$), serum insulin ($P < 0.001$) and IR ($P < 0.001$). Interestingly, blood glucose and serum insulin levels in CTRL + Q were observed to be nonsignificantly lower than CTRL.

Behavioral studies

Effect of quercetin treatment on CUS induced behavioral dysfunction was evaluated during 22-28 days of the study. Locomotor activity was evaluated using Actophotometer and OFT. Muscle coordination was evaluated by using Rota-rod apparatus and beam walk test. Anxiety level in experimental animals was evaluated through EPM and OFT. Depressive behavior was evaluated through SPT, TST and FST. Effect of quercetin on STZ-induced diabetes on memory dysfunction was evaluated through PA-SD test, PA-ST test, NOR test and MWM test (74).

Chronic stress resulted in reduced locomotion and development of anxiety in animals. Number of line crossings in Actophotometer and OFT were significantly ($P < 0.001$) reduced. Animals were highly anxious, spent significantly lesser duration in the open arm of EPM and in the central region of OFT, besides, significantly ($P < 0.001$) lower entries to the central region of OFT. Results of Rota-rod and beam walk revealed that stressed animals were having normal muscle coordination and 21 day CUS do not have any significant effect on muscle coordination. These results suggest that observed locomotor impairment may have central origin as a result of CUS since muscle coordination and strength was observed to be normal in all the treatment groups.

We further evaluated effect of quercetin on CUS mediated depressive like behavior. We observed that CUS resulted in significant ($P < 0.001$) anhedonia, and animals showed lack of interest towards rewarding stimuli. Depressive like behavior in stressed animals was confirmed by TST and FST, where immobility time was observed to be significantly ($P < 0.05$) higher than CTRL. Treating animals with quercetin alleviated stress mediated anhedonia and significantly ($P < 0.001$) reduced immobility duration during TST and FST. These results suggest that quercetin is having a strong anti-depressant potential and efficiently alleviated stress mediated depression.

We further investigated the effect of quercetin treatment on short term and long term memory retention through PA-ST and PA-SD task. Stressed animals showed significantly ($P < 0.001$) impaired memory performance in PA-SD and PA-ST task. Memory acquisition training was given on day 1 and memory retention was evaluated on day 2 (short term memory) and day 5 (long term

memory) in terms of transfer latency. CUS resulted in significantly ($P < 0.001$) reduced transfer latency in both the model on day 2 as well as day 7, indicating impaired memory retention. Quercetin treatment efficiently alleviated memory dysfunction and took longer duration to enter grid platform. We further evaluated the effect of quercetin treatment on alterations in recognition memory using NOR test. CUS animals spent approximately equal time exploring both, novel and familiar objects, indicating that they were not able to discriminate familiar object from novel one. Discrimination index was significantly ($P < 0.001$) reduced in stressed animals, which was significantly ($P < 0.001$) improved after 21 day quercetin treatment. Further, we evaluated the effect of quercetin treatment on stress mediated learning and spatial memory dysfunction using MWM test. On day one of training, all the animals struggled to find the hidden platform and transfer latency did not differed significantly in all the treatment groups. Effect of CUS on learning abilities of animals become evident from second day of training where stressed mice showed significantly ($p < 0.05$) higher transfer latency when compared to CTRL. On day 3 and day 4 also, transfer latency in CUS group were observed to be significantly ($p < 0.01$) higher than CTRL group. Quercetin treated animals showed improved learning across the test and transfer latency was observed to be significantly ($P < 0.01$) lower than CUS and were comparable to CTRL. We further evaluated the memory index in term of total time spent in the platform quadrant and number of platform crossings. Results revealed the CUS animals spent significantly ($P < 0.01$) lower time in platform quadrant and showed significantly ($P < 0.01$) lower number of platform crossings. Quercetin treated animals showed good memory index and animals spent significantly higher time in platform quadrant and crossed platform position frequently in search of escape platform. These results suggests that quercetin can efficiently rescue animals from stress mediated memory dysfunction.

Animal sacrifice and sample collection

Samples for the biochemical evaluations were collected 24 h after the last behavioral test. Animals were sacrificed under anesthesia (90 mg/kg ketamine and 5 mg/kg xylazine) and entire brain was either fixed in 2% paraformaldehyde and 2% glutaraldehyde solution (for histopathological and immunofluorescence studies) or used as such for Golgi-cox staining. For biochemical and proteomics studies, animals were sacrificed by cervical dislocation, hippocampus was dissected over ice, homogenized in RIPA buffer and stored at -80ºC.

Biochemical evaluation

Effect of quercetin treatment on hippocampal oxidative stress markers was evaluated in the prepared hippocampal homogenate in terms of TTH (412 nm), TBARS (540 nm), NO (540 nm) and catalase levels (240 nm) as per our previously described method (75). CUS resulted in significant ($P < 0.001$) reduction in hippocampal TTH and catalase levels, which are essential to neutralize ROS/RNS and protect neurons from oxidative damage. Further, CUS significantly ($P < 0.001$) increased the expression of TBARS and NO levels in the hippocampal tissue, suggesting an elevation in oxidative stress. Quercetin is a natural antioxidant and it efficiently upregulated the levels of antioxidants (TTH and catalase) and significantly ($P < 0.001$) reduced hippocampal oxidative stress markers. These results reconfirms our findings in STZ model that quercetin have high potential to alleviate hippocampal oxidative stress.

Neurodegeneration

Effect of quercetin on CUS induced hippocampal neurodegeneration was evaluated through histopathological examination using hematoxylin-eosin and cresyl violet staining (76, 77). Our results mimic the findings of objective 2 and demonstrated that CUS inflicts severe damage in the CA3 region of the hippocampus. Neurons appeared degenerated, especially in the CA3 region of the hippocampus. 21 day quercetin treatment rescued neurons from stress mediated damage and neurons resembled normal morphology, suggesting the neuroprotective potential of quercetin.

Protein expression studies

In order to get a better insight into the effect of IR and quercetin treatment on hippocampal insulin signaling, protein expression in the hippocampus was determined through western blot analysis (30). CUS induced IR resulted in significant ($P < 0.001$) reduction of InR and GLUT4 expression in the hippocampus. 21 day quercetin treatment upregulated the hippocampal expression of InR and GLUT4. Interestingly, normal animals treated with quercetin showed significantly reduced InR and significantly higher GLUT4 expression, when compared to CTRL. Despite of higher insulin levels in stressed animals, these finds suggest that CUS induces insulin resistant state in hippocampus and quercetin upregulate neuronal insulin signaling which seems to be independent of insulin and InR. Further, in order to get a better insight, we performed immunofluorescence studies. Our results demonstrated that expression of InR and GLUT4 was significantly ($P < 0.001$) reduced in hippocampus during CUS. Quercetin treatment in both normal and stressed animals

resulted in enhanced expression of InR and GLUT4. Further, since number of viable cells in the hippocampus of stressed animals were lower, we calculated expression of InR and GLUT4 in the neurons of CA3 region (where maximum damage was observed). We determined ratio of GLUT4:InR expression since activation of InR leads to upregulation of GLUT4 expression or in other words expression of InR indirectly correlates to GLUT4. Interestingly these was no significant difference in the InR and GLUT4 expression on viable neurons of CA3 region in CUS animals, when compared to CTRL. However, a significant ($P < 0.05$) reduction in InR expression and significant ($P < 0.01$) elevation in GLUT4 expression was observed in animals treated with quercetin (CTRL + Q and CUS + Q), when compared to CTRL or CUS. GLUT4:InR ratio also demonstrated that there is no significant difference in CUS and CTRL, however, CTRL + Q and CUS + Q varied significantly ($P < 0.001$) from CTRL and CUS. These findings suggest that quercetin have potential to modulate insulin signaling, in both normal and insulin resistant state, and its mechanism of action is independent of the expression of InR.

Conclusion

Our results demonstrated that CUS induced IR and pre-diabetic state in mice. CUS mediated IR was associated with behavioral dysfunction which was attributed to enhanced hippocampal oxidative stress, reduced hippocampal neurogenesis and impaired insulin signaling pathway. Quercetin treatment attenuated IR, normalized blood glucose levels, and alleviated CUS mediated behavioral dysfunctions. Beneficial effects of the quercetin were attributed to its antioxidant, neuroprotective and insulin signaling modulating potential. Quercetin improved neurogenesis and upregulated neuronal GLUT4 expression which were observed to be independent of insulin and InR expression. Quercetin may find a clinical application in the management of T2DM and IR and associated neurological complications, which need further investigation.

Objective 4

To elucidate the mechanism through which quercetin modulates glucose homeostasis

In-vitro studies to confirm the mechanism of action of quercetin was carried out on SH-SY5Y neuroblastoma cell line. Confluent cell culture was starved for 24 h by incubating it with glucose free media in a 96-well culture plate. Neurons were then incubated with 1 µM LY294002 (insulin signaling inhibitor) for 20 min and then treated with 10 µM quercetin for 15 min at 37 ºC. Cells were then incubated with 0.1 mM 2-NBDG (fluorescent glucose analog) for 15 min at 37 ºC. Neuronal culture was washed and amount of glucose uptake in the presence and absence of LY294002 and the effect of quercetin treatment was determined in terms of fluorescence intensity of 2-NBDG in the neuronal cultures. Our results demonstrated that treating neurons with LY294002, LY294002 + quercetin and LY294002 + insulin showed significantly lower glucose uptake, when compared to control cells. However, treating neurons with quercetin and insulin alone resulted in enhanced glucose uptake and results were significantly higher than control and LY294002 treated neurons. These results confirms that intact insulin signaling pathway is essential for quercetin activity and it can upregulated insulin signaling independently of insulin and therefore resulted in enhanced glucose uptake in normal cells.

Further, we predict the interaction site/active site of quercetin on InR using Metapocket server. Our results suggest that quercetin is having strong interaction with the InR, however, binding site of quercetin was found to be different than site used by insulin to activate InR and downstream signaling. Interaction of quercetin with InR was observed at Thr293, Lys1165, Gly1169, Glu314, Arg212, Leu1171, Arg1136, Asp1132 (hydrogen bond); Ala291, Leu5, Arg20, Glu294, Asn1215, Tyr312, Val1173, Lys1168, Ala315, Ser4, Ile6, Trp1175 (hydrophobic interaction). Interaction of insulin with InR was observed at Asp1132, Arg1136, Gly1169, Lys1165, Met11, Met13, Tyr10, Asp9, Gly8, Leu15, Ala14 (hydrogen bond); Gly20, Leu6, Ile2, Val12, Tyr26, Glu1004 (hydrophobic interaction).

Conclusion

Through *in-vitro* fluorescence glucose (2NBDG) assay we confirmed that quercetin activates neuronal insulin signaling pathway. Both insulin and quercetin enhanced glucose uptake into the neuronal cells, however, they failed to enhance uptake when neurons were treated with LY294002, which is a specific inhibitor of insulin signaling pathway, suggesting that quercetin is capable of

activating neuronal insulin signaling. Moreover, results of the *in-silico* docking studies suggest that quercetin interact strongly with the InR and the binding site is different from that used by insulin to activate downstream signaling.

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